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[Original]

Inhibitory Effect of Anti-Candida Substance Produced by *Bacillus natto*

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Abstract

Bacillus natto produced antifungal substance extracelluraly which inhibited the growth of *Candida albicans* and *C. stellatoidea*, but did not inhibit the growth of other Candida species. This antifungal substance was obtained from the culture filtrate by a procedure including the addition of active carbon and molecular filtration (MW 10,000), and this substance was designated as crude Anti-Candida substance (ACS). By paper disk method with crude ACS, the inhibitory zone was detected in all strains of *C. albicans* but not detected in any strains of *C. tropicalis*. A clear distinguishment between these two species was obtained by this method. MIC of crude ACS against *C. albicans* was 400 μg/ml but *C. tropicalis* growth was not inhibited at 12,800 μg/ml of crude ACS. Crude ACS which was stored at -20°C and crude ACS solution which was stored at 4°C or -20°C retained its original inhibitory activity for 8 weeks. The action of crude ACS against *C. albicans* was mainly bacteriostatic and weakly bacteriocidal. This result was also identical with the morphological aspect of *C. albicans* treated with crude ACS. Regarding the morphological aspects of *C. albicans* on crude ACS-Sabouraud's dextrose agar plate some organisms were swollen at first, and as a secondary step these were destroyed.

In conclusion, this study suggests that a more easy, rapid, and exact identification of *C. albicans* can be done by the utilization this paper disk method in parallel with other identification methods.

Key words : *Candida albicans*, *Bacillus natto*, Anti-Candida substance

Introduction

Many Candida species are detected in the human body, such as *Candida albicans*,

C. tropicalis, *C. pesudotropicalis*, *C. krusei*, *C. parapsilosis*, *C. stellatoidea*, and *C. guillermondii*. Especially, *C. albicans* is known to be one of the major opportunistic pathogen that frequently caused superinfection[1]. From the pathogenecity, it is important to identify which organisms are isolated from the patient. To identify these species, there are many identification methods. But very few reports on easy identification of *C. albicans* are available, because the morphology and growth are complex, and in addition the fermentation and utilization of carbohydrates are unstable in comparison with other bacteria.

On the other hand, *Bacillus subtilis* produced various antifungal substances, such as Bacillomycin, Fungistatin, and Mycobacillin etc[2]. Kobayashi[3,4] reported that *B. subtilis* produced an antifungal substance which strongly inhibited the growth of *C. albicans*, but failed to inhibit the growth of *C. tropicalis*. Further, she suggested that this character could be applied to the identification of Candida species. The antibiotic effect of *B. natto* has also been reported by Mori and Okamoto[5], Ishidate et al[6], Ushijima and Ozaki[7], and Ozawa et al[8]. But no mention was made the selective inhibitory effect against Candida species.

In this study, a crude Anti-Candida substance (ACS) was prepared from the culture filtrate of *B. natto*, and was examined for the selective inhibitory effect against Candida species. The stability at storage conditions, function of crude ACS and morphorogical aspect of *C. albicans* which was exposed to crude ACS were examined. The main object of this study is to examine whether the characteristic of crude ACS is useful for identification of *C. albicans*, or not.

Materials and methods

Strains. Eight strains of *B. natto* were isolated from fermented soybeans known as "natto". The biological characteristics of these strains are shown in Table 1. Candida species which were used in this experiment are shown in Table 2. All Candida species maintained as stock culture at our laboratory.

Culture conditions. *B. natto* strains were cultured in nutrient agar slant (Eiken Chemical Co., Ltd) at 37°C or Kobayashi's soybean-starch medium[4] (hot extract of 12.5 % soybean, 1 % starch, pH5.8) at 25°C. All Candida species were cultured on Sabouraud's dextrose agar (SDA) slant (Eiken Chemicals Co., Ltd), plate or broth at 37°C. PT medium (1 % peptone and 1 % tween 80) was used for forming the mycelium of *C. albicans* and incubated at 37°C. Except where mentioned especially, most cultures were carried out under static conditions. Shaking culture was carried out with water bath incubator Model BT-46 (Yamato Kagaku Co., Ltd).

Preparation of crude ACS. Crude ACS was prepared by a modification of the procedure described by Kobayashi[3,4]. *B. natto* strain NA-6 was precultured on nutrient agar slant

Table 1 Biological characteristics of *B. natto* strains

Character	Strains tested							
	NA-I	NA-2	NA-3	NA-4	NA-5	NA-6	NA-7	NA-8
Gram staining	+	+	+	+	+	+	+	+
Shape	rod	rod	rod	rod	rod	rod	rod	rod
Spore	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+
O-F test	0	0	0	0	0	0	0	0
Catalase	+	+	+	+	+	+	+	+
Pigment	+	+	+	+	+	+	+	+
Indol	-	-	-	-	-	-	-	-
Gelatinase	+	+	+	+	+	+	+	+
Milk digestion	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-
DNase	-	-	-	-	-	-	-	-
Lecithinase	-	-	-	-	-	-	-	-
Tween 80 hydrolysis	-	-	-	-	-	-	-	-
Acid from carbohydrate								
Glucose	+	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-
Dextrin	-	-	-	-	-	-	-	-
Mannite	+	+	+	+	+	+	+	+
Gas productivity	-	-	-	-	-	-	-	-
Acid from glycogen	+	+	+	+	+	+	+	+
Complete hydrolysis of								
starch	+	+	+	±	+	+	+	+
amylose	+	+	+	±	+	+	+	+
Utilization of histidine	+	+	+	±	+	+	+	+

at 37°C for 16 hr and then the organism was inoculated into 2 ℓ of soybean-starch medium. The cultivation was carried out at 25°C for 6 days. The medium was centrifuged at 8,000 rpm for 30 min, and the supernatant was adjusted to pH 2.3 with 10% HCl. Active carbon was added to the supernatant to give 0.5%, then stirred for 30 min with a magnetic mixer. After the solution was filtrated with 0.45 μ membrane filter (Toyo Roshi Co., Ltd), the filtrate was adjusted to pH 5.6 with 2N-NaOH. The solution was filtrated with ultra-filter (MW 10,000, Millipore USA), and then the filtrate was concentrated to one-tenth of its original volume in vacuum at 35°C. The concentrated solution was lyophilized and designated as crude ACS.

Assay of inhibitory activity. (1) Paper disk method. Candida species were precultured on SDA slant at 37°C for 24 hr and then one loopful of the organism was suspended in a saline. 0.1 ml of the cell suspension was spread onto SDA plate. A paper disk immersed in crude ACS (128 mg/ml) was placed onto the plate. After the plate was incubated at 37°C for 48 hr, the inhibitory activity was indicated by its inhibitory zone diameter.

Table 2 Candida species employed

Species	Strains	Source
C.albicans	ATCC 1012, ATCC 1002, 1011, 401, FIA 1001, Basel A, Basel B, Duke 1001, MTU 12013, Ozawa, Okabe.	standard strains
	KC 98	old stock culture isolated from oral candidiasis at Kyushu Dental College
	E92, E106.	fresh isolates from oral candidiasis at Higashi-Nippon-Gakuen University
	C1, C2, C3, C4, C5, C6, C7, C10, C11, C12, C14, C15, C16, C17, C18, C23, C25, C26, C27, C29 C30, Kla.	old stock cultures isolated from skin candidiasis at Eto clinic
	E1, E2, E3, E4, E8, E9, E10, E12, E13, E14, E15, E16, E17, E18, E19, E20, E21, E22, E23, E24, E25, E26, E27, E28, E29, E30, E31, E32, E36, E37, E38, E39, E40, E41, E42, E43, E44, E45, E47, E49, E50, E51, E53, E54, E56, E57, E58, E59, E60, E61, E62, E63, E64, E65, E66, E67, E68, E69, E70, E71, E72, E73, E74, E75, E76, E77, E78, E79, E80, E81, E82, E83, E84, E85, E86, E87, E88, E89, E90, E91 E93, E94, E95, E96, E99, E100, E102, E103, E104, E105, E107, E108, E109, E110, E112, E113, E114, E115, E117, E118, E119, E120, E121, E122, E123, E124, E125, E126, E127, E128, E129, E130, E131, E132, E133, E134.	fresh isolates from skin candidiasis at Eto clinic
C.tropicalis	403, ATCC 1003, 1017, 1113, 7397.	standard strains
C.pseudotropicalis	426, 1004, 1026, 7494.	standard strains
C.stellatoidea	427, 1027, 1223, 1361, 1362.	standard strains
C.krusei	427, IFO 1012, 4024.	standard strains
C.parapsilosis	1015, 1025.	standard strains
C.guilliermondii	582 OUT, 1007, 1023, 1121.	standard strains

(2) Dilution method. One ml of crude ACS solution (12.8 mg/ml) was diluted two-fold with Sabouraud's dextrose (SD) broth continuously. 0.1 ml of cell suspensions (10^6 cells/ml) of Candida species were inoculated into serial diluted crude ACS-SD broth and SD broth (control). After incubation at 37°C for 24 hr, the minimal inhibitory concentration (MIC) of crude ACS against Candida species were determined by measuring its turbidity macroscopically.

Measurement of growth and viable cell count. Growth of *C. albicans* Kla was measured by optical density at 540 nm with Hitachi Model 100-10 spectrophotometer and viable cell count was measured by macroscopic observation of colony grown on SDA plate. Survivals in crude ACS-SD broth were determined with samples which were picked up at various periods of incubation times. 0.1 ml of samples which were diluted ten-fold continously were imediately spread onto SDA plates. After 48 hr of incubation, visible colonies were counted.

Morphology. (1) On crude ACS-SDA plate. *C. albicans* Kla was precultured on SDA slant at 37°C for 24 hr and one loopful of the organism was suspended in a saline. A drop of the cell suspension was dropped onto crude ACS-SDA plate and its morphology was observed under light microscope directly. The plate was incubated at 37°C for 24 hr.
(2) In crude ACS-SD broth. *C. albicans* Kla was precultured on SDA slant at 37°C for 24 hr and one loopful of the organism was added into 10 ml of crude ACS-SD broth, and then the broth was incubated at 37°C for 24 hr. A control of the organism which was inoculated into SD broth was prepared. The organism was harvested by centrifugation at 3,000 rpm for 15 min and then stained with Gram stain method.
(3) In crude ACS-PT medium. *C. albicans* Kla was precultured on SDA slant and about 10^3 cells of the organism were inoculated into crude ACS-PT medium. A control of the organism was inoculated into PT medium. The medium was incubated at 37°C for 48 hr. The organism was harvested by centrifugation at 3,000 rpm for 15 min and then stained with Gram stain method.

Cellulose thin layer chromatography (Cellulose-TLC). Crude ACS was applied to cellulose thin layer plate (Merck) and developed at room temperature for 6 hr in the following solvent : n-butanol + acetic acid + water (4+1+1). After the developed plate was dried, guide strip (20mm) was cut off and the strip was sprayed with ninhydrin solution. Concurrently, other none sprayed plates were divided into ten fractions and those fractions were scraped off from the plate. Those fractions were placed on SDA plate to which was added the *C. albicans* Kla cell suspension. The plate was incubated at 37°C for about 24 hr until clear none-growing spots owing to crude ACS appeared on the plate.

Results

1. Differences of inhibitory effect

Eight strains of *B. natto* were precultured on nutrient agar slant at 37°C for 16 hr and then one loopful of the organism was inoculated onto SDA plate containing living cell suspensions of *C. albicans* Kla or *C. tropicalis* ATCC 1017. As shown in Fig. 1, a clear inhibitory zone was formed around *B. natto* colonies against *C. albicans* Kla, but not against *C. tropicalis* ATCC 1017. From this result, it was considered that *B. natto* produced a selective inhibitory substance against Candida species extracellulary.

To select the most strong productive strains of *B. natto*, these strains were cultivated with soybean-starch medium. The inhibitory activity was measured with the dilution method. As shown in Table 3, the culture filtrate of *B. natto* clearly inhibited the growth of *C. albicans* Kla, and strain NA-1, NA-3, NA-6, NA-7 and NA-8 gave a 2- to 4- fold higher degree of inhibitory activity than strain NA-2, NA-4 and NA-5. NA-6 strain was used throughout this experiment for production of inhibitory substan-

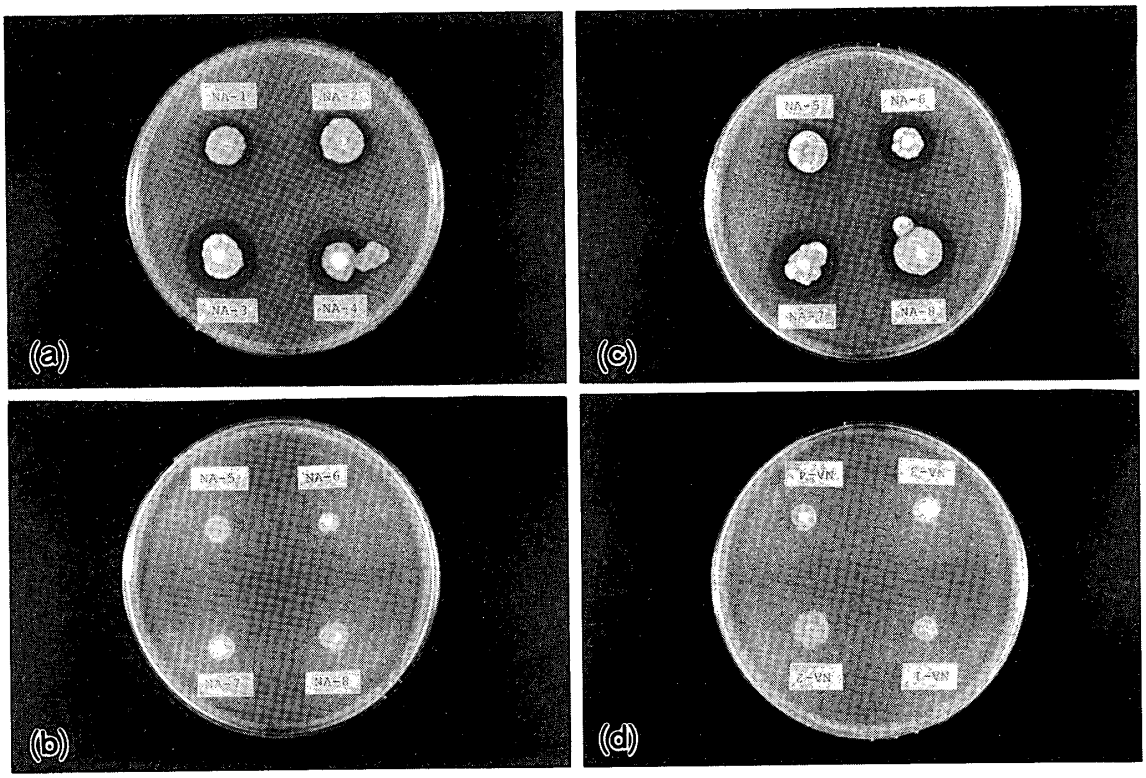

Fig. 1 Differences on the inhibitory effect of 8 strains of *B. natto* against *C. albicans* and *C. tropicalis*. (a) and (b) contain *C. tropicalis* ATCC 1017 living cells. (c) and (d) contain *C. albicans* Kla living cells. Other procedures are described in "Materials and methods".

Table 3 Inhibitory activity of culture filtrate from various strains of *B. natto* against *C. albicans* Kla

	Activity									
dilution fold / strain	2 X	4 X	8 X	16 X	32 X	64 X	128 X	256 X	518 X	control
NA-1	-	-	-	-	-	-	+	++	++	+++
NA-2	-	-	-	-	±	+	++	++	+++	+++
NA-3	-	-	-	-	-	-	+	++	++	+++
NA-4	-	-	-	-	-	+	++	++	+++	+++
NA-5	-	-	-	-	-	+	++	++	+++	+++
NA-6	-	-	-	-	-	-	±	+	++	+++
NA-7	-	-	-	-	-	-	+	++	++	+++
NA-8	-	-	-	-	-	-	+	++	++	+++

Eight strains of *B. natto* were precultured on nutrient agar slant at 37°C for 16 hr and then individually one loopful of the organisms were inoculated into 100 ml of soybean-starch medium. The organisms were incubated at 25°C for 6 days, and 10 ml of the culture fluid were picked up. After the culture fluid was centrifuged at 8,000 rpm for 30 min, the supernatant was filtrated with 0.45 μ membrane filter. The inhibitory activities of culture filtrates of *B. natto* were estimated by dilution method.

Remarker : +++ ; very good growth, ++ ; good growth, + ; moderate growth, ± ; poor growth, − ; no growth.

ce, because it was regarded that strain NA-6 indicated only a slightly higher activity than other strains.

On the contrary, *C. tropicalis* ATCC 1017 was not quite inhibited by culture filtrate of *B. natto* (data not shown). It was shown that there was a clear difference in inhibitory activity of culture filtrate of *B. natto* against *C. albicans* and *C. tropicalis*.

2. Inhibitory activity with paper disk method

Crude ACS was prepared as described in "Materials and methods" and separated as a light-brown mass. To confirm the selective inhibitory activity of crude ACS against Candida species, inhibitory tests were performed with the paper disk method (Fig. 2).

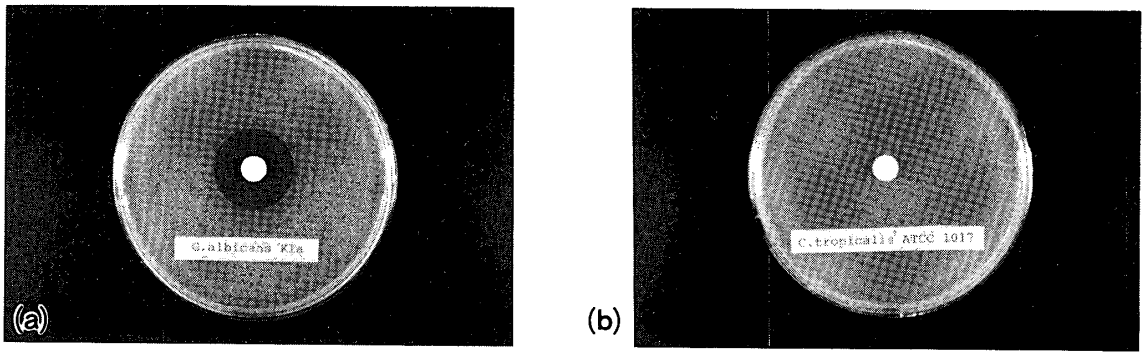

Fig. 2 Demonstration of the inhibitory effect by paper disk method with crude ACS against *C. albicans* and *C. tropicalis*. Procedures are described in "Materials and methods".

The results of these are shown in Table 4. 145 strains of *C. albicans* and only one strain of *C. stellatoidea* were inhibited markedly (in symbol ; +4). 4 strains of *C. albicans* were inhibited remarkably (+3). 5 strains of *C. albicans* and one strain of *C. stellatoidea* were inhibited intermediately (+2). One strain of *C. stellatoidea* was inhibited weakly (+1). Many strains of *C. tropicalis*, *C. pseudotropicalis*, *C. krusei*, *C. parapsilosis*, and *C. guilliermondii* were not quite inhibited with crude ACS. Therefore it was clarified that *C. albicans* and *C. stellatoidea* were inhibited with crude ACS, but other species of Candida were not. This result indicated that there was a difference in susceptibility toward crude ACS among Candida species.

3. MIC of crude ACS

MIC of crude ACS against Candida species were examined by the dilution method as described in "Materials and methods". MIC of crude ACS against *C. albicans* ATCC 1012, Basel A, Basel B and Kla was 400 μg/ml, and against 4 strains of *C. stellatoidea* were 800 μg/ml, 1,600μg/ml (two strains) and above 12,800 μg/ml respectively. *C. albicans* was more susceptible to crude ACS than *C. stellatoidea*. With the exception of *C. guilliermondii* 1121, other Candida species were not inhibited at 12,800 μg/ml of crude ACS (Table 5). These results were similar to that of the paper disk method.

Table 4 Inhibitory activity of crude ACS on growth of Candida species

Inhibitory activity	Strains
4+	*C.albicans* ATCC 1012, 1011, FIA 1001, Basel A, Basel B, Ozawa, K1a, KC98, C1, C2, C3, C4, C5, C6, C7, C10, C12, C14, C15, C16, C18, C23, C25, C26, C27, C29, C30, E1, E2, E3, E4, E8, E10, E12, E13, E14, E15, E16, E17, E19, E20, E21, E22, E23, E24, E25, E26, E27, E28, E29, E30, E31, E32, E36, E37, E38, E39, E40, E41, E42, E43, E44, E45, E47, E49, E50, E51, E53, E54, E56, E57, E58, E59, E60, E61, E62, E63, E64, E65, E66, E67, E68, E69, E70, E71, E72, E73, E74, E75, E76, E77, E78, E79, E80, E81, E82, E83, E84, E85, E86, E87, E88, E89, E90, E91, E93, E94, E95, E96, E97, E98, E99, E100, E102, E103, E104, E105, E107, E108, E109, E110, E112, E113, E114, E115, E117, E118, E119, E120, E121, E122, E123, E124, E125, E126, E127, E128, E129, E130, E131, E132, E133, E134.
	C.stellatoidea 1361
3+	*C.albicans* Duke 1001, ATCC 1002, Okabe, C11.
2+	*C.albicans* MTU 12013, 401, C17, E9, E18.
	C.stellatoidea 1362.
1+	*C.stellatoidea* 427, 1027.
	C.stellatoidea 1223.
	C.tropicalis ATCC 1003, 1017, 1113, 403, 7397.
	C.pseudotropicalis 426, 1004, 1026, 7494.
	C.krusei IFO 1012, 427, 4024.
	C.parapsilosis 1015, 1025.
	C.guilliermondii 1007, 1023, 1121, 582 OUT.

Inhibitory activities were designated by inhibitory zone diameter as following ; 4 + ≧22mm, 21≧ 3 + ≧18mm, 17≧ 2 + ≧14mm, 13≧ 1 + ≧10mm, 9 mm≧ −.

4. Stability at various temperatures

Stability of crude ACS was examined at various storage temperatures. As shown in Fig. 3, crude ACS which stored in a desicator at room temperature rapidly decreased its activity, and crude ACS solution (12.8 mg/ml) stored at 37°C was stable until 5 days, but over 7 days, its activity decreased. The activities of crude ACS stored at −20°C and crude ACS solution stored at 4°C or −20°C were stable for 8 weeks and crude ACS of these storage conditions maintained three quarter activity until 13 weeks. Therefore, it was decided that storage at −20°C was the optimal condition for stability of crude ACS.

5. Morphological change

Morphological changes of *C. albicans* Kla exposed to crude ACS were examined in three ways ; i. e. on crude ACS-SDA plate, in crude ACS-SD broth, and in crude ACS-PT medium. On the crude ACS-SDA plate, it was observed that some of the organisms

Table 5 MIC of crude ACS against various species of Candida

Species	MIC (μg/ml)
C.albicans ATCC 1012	400
C.albicans Basel A	400
C.albicans Basel B	400
C.albicans Kla	400
C.stellatoidea ATCC 1027	1,600
C.stellatoidea 427	1,600
C.stellatoidea 1223	>12,800
C.stellatoidea 1361	800
C.tropicalis ATCC 1017	>12,800
C.tropicalis ATCC 1003	>12,800
C.pseudotropicalis 1026	>12,800
C.krusei IFO 1012	>12,800
C.parapsilosis 1025	>12,800
C.guilliermondii 1023	>12,800
C.guilliermondii 1121	6,400
C.guilliermondii 1007	>12,800
C.guilliermondii 582 OUT	>12,800

Procedures are described in "Materials and methods".

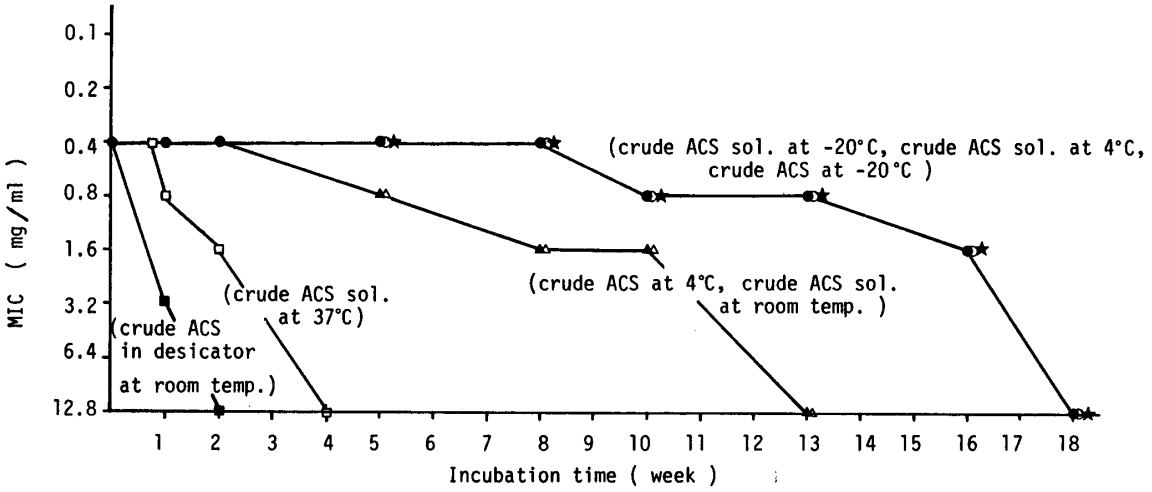

Fig. 3 Stability of crude ACS and crude ACS sol. at various storage temprature. Crude ACS sol. (12.8 mg/ml) was sterilized with 0.45 μ membrane filter and it was stored in a pre-heated sterilized test tubes at various temperatures. Crude ACS mass was stored in test tubes at various temperatures. Samples were taken at various times for measurement of MIC. Symbols : ■ ; crude ACS in desicator at room temerature, □ ; crude ACS sol. at 37°C, ▲ ; crude ACS at 4°C, △ ; crude ACS sol. at room temperature, ● ; crude ACS sol. at −20°C, ○ ; crude ACS sol. at 4°C, ★ ; crude ACS at −20°C. Other procedures are described in Materials and Methods.

swelled after 3 hr (Fig. 4a) and destruction-like phenomenon occured after 24 hr (Fig. 4b). Even after 24 hr, no colony was detected on the plate. In crude ACS-SD broth, a cytoplasm-like substance was observed around the cells and many cells changed to Gram

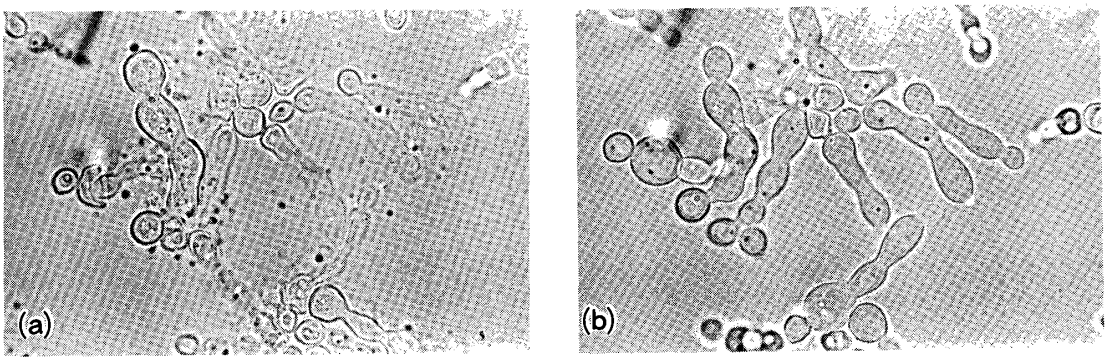

Fig. 4 Morphological changes of crude ACS treated *C. albicans*.
(a) : Photomicrograph of cells of *C. albicans* Kla on crude ACS (800 μg/ml)-SD plate after 3 hr. (X 1,000)
(b) : Photomicrograph of cells of *C. albicans* on the same plate after 24 hr. (X 1,000)

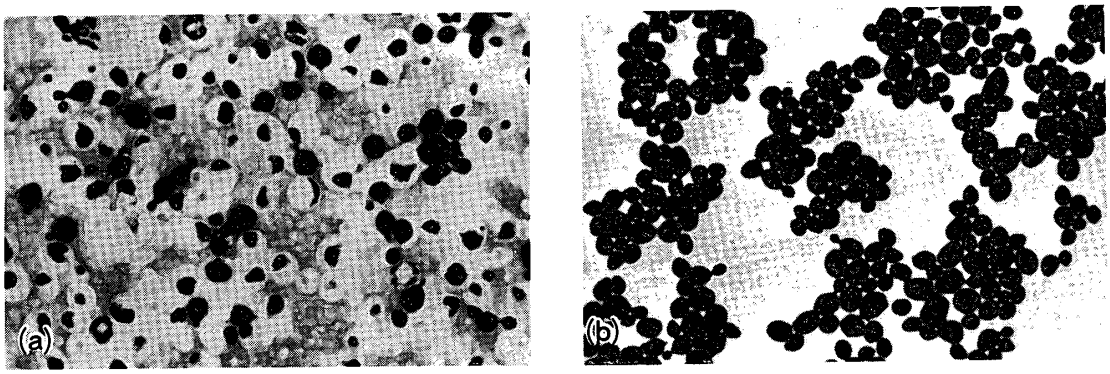

Fig. 5 Morphological changes of crude ACS treated *C. albicans*.
(a) : Photomicrograph of cells of *C. albicans* Kla in crude ACS (1,600 μg/ml)-SD broth after 24 hr. (X 1,000)
(b) : Photomicrograph of cells of *C. albicans* Kla in SD-broth. (X 1,000)

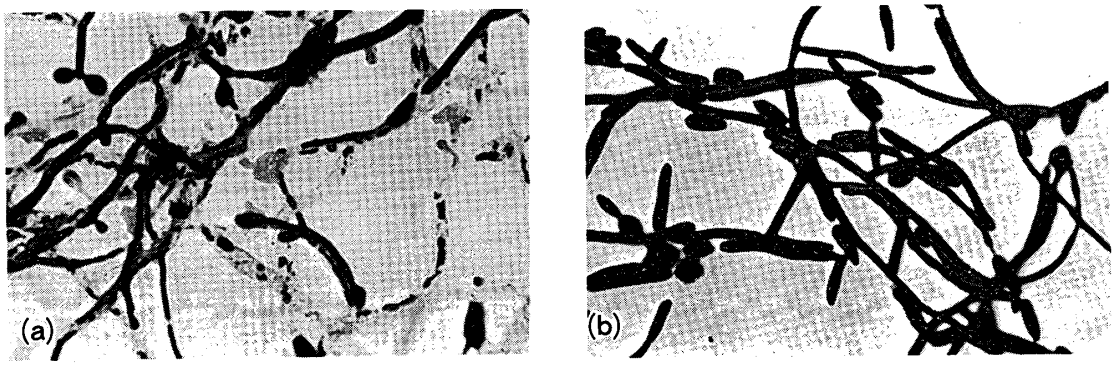

Fig. 6 Morphological changes of mycelium of *C. albicans* in crude ACS-PT broth.
(a) : Photomicrograph of cells of *C. albicans* Kla in crude ACS (4,000 μg/ml)-PT broth after 24 hr. (X 1,000)
(b) : Photomicrograph of cells in PT-broth. (X 1,000)

negative (Fig. 5a). In crude ACS-PT medium, many mycellium type of *C. albicans* were also changed to Gram negative (Fig. 6a). It was thus observed that both yeast and mycellium type of *C. albicans* were susceptible to crude ACS.

6. Function

To study the function of crude ACS against *C.albicans*, the growth and number of viable cells of *C. albicans* Kla was examined. When crude ACS was added at a lag-phase, after 60 min, the growth was inhibited and viable cells decreased from 1×10^5 to 2.2×10^2 cells/ml after 8 hr (Fig. 7a, b). When crude ACS was added at an early log-phase, after 60 min, the growth was also inhibited and viable cells decreased from 5.5×10^5 to 3.1×10^3 cells/ml after 8 hr (Fig. 8a, b). All of the cells, however, were not killed by crude ACS, and it was proved by the morphological aspects and the fact that viable cells increased in both cases after 24hr. It may be attributed to the existence of resistance cells against crude ACS. From these results, it was assumed that the function of crude ACS against *C. albicans* was bacteriostatic and weakly bacteriocidal.

7. Cellulose-TLC

As shown in Fig. 9, the ninhydrin positive six bands were observed on cellulose-TLC and inhibitory activity was observed in fraction No.7 strongly, fraction No.8 weakly, and fraction No.6 very weakly. Fraction No.7 and No.8 coincided with a ninhydrin positive bands, but the fraction No.6 was in the ninhydrin negative portion.

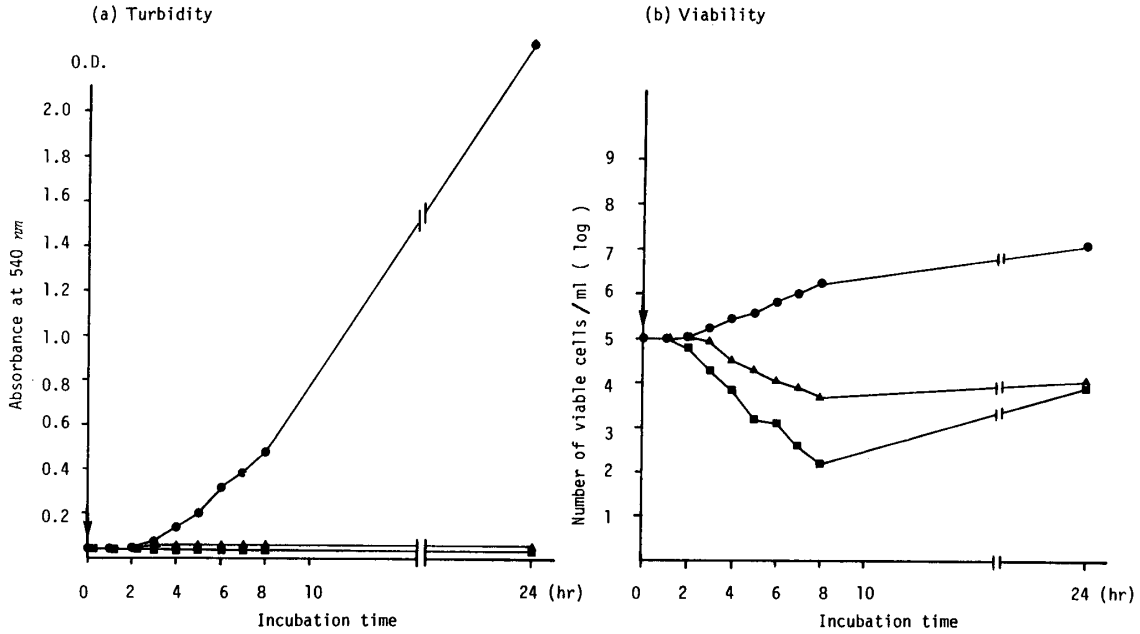

Fig. 7 Action of crude ACS against *C. albicans*. An exponential growing culture of *C.albicans* in SD broth was diluted with fresh medium to give an absorbance at 540 *nm* of 0.30 and then 1 ml of cell suspension was placed in several test tubes (1.8 × 90mm) containing 8 ml of SD broth to be tested. At lag-phase (0 hr), 1 ml of crude ACS (■ : 128 mg/ml, ▲ : 4 mg/ml) -SD broth and 1 ml of SD broth (● : control) were added to each tube. The test tubes were incubated for 24 hr with constant shakig 37°C. Samples were taken at various times for measurement of growth (a) and viable count (b). Solid arrow indicates crude ACS addition.

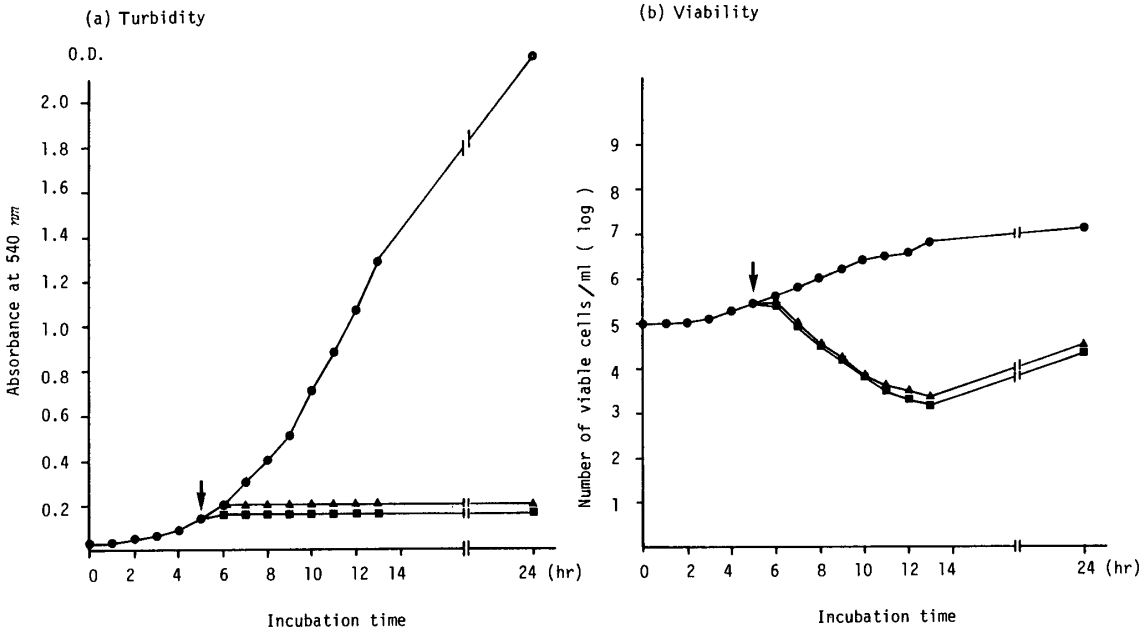

Fig. 8 Action of crude ACS against *C. albicans*. At early log-phase (5 hr), 1 ml of crude ACS-SD broth (■ : 128 mg/ml, ▲ : 4 mg/ml) and 1 ml of SD broth (● : control) were added to each tube. Other procedures described in Fig. 7. Solid arrow indicates crude ACS addition.

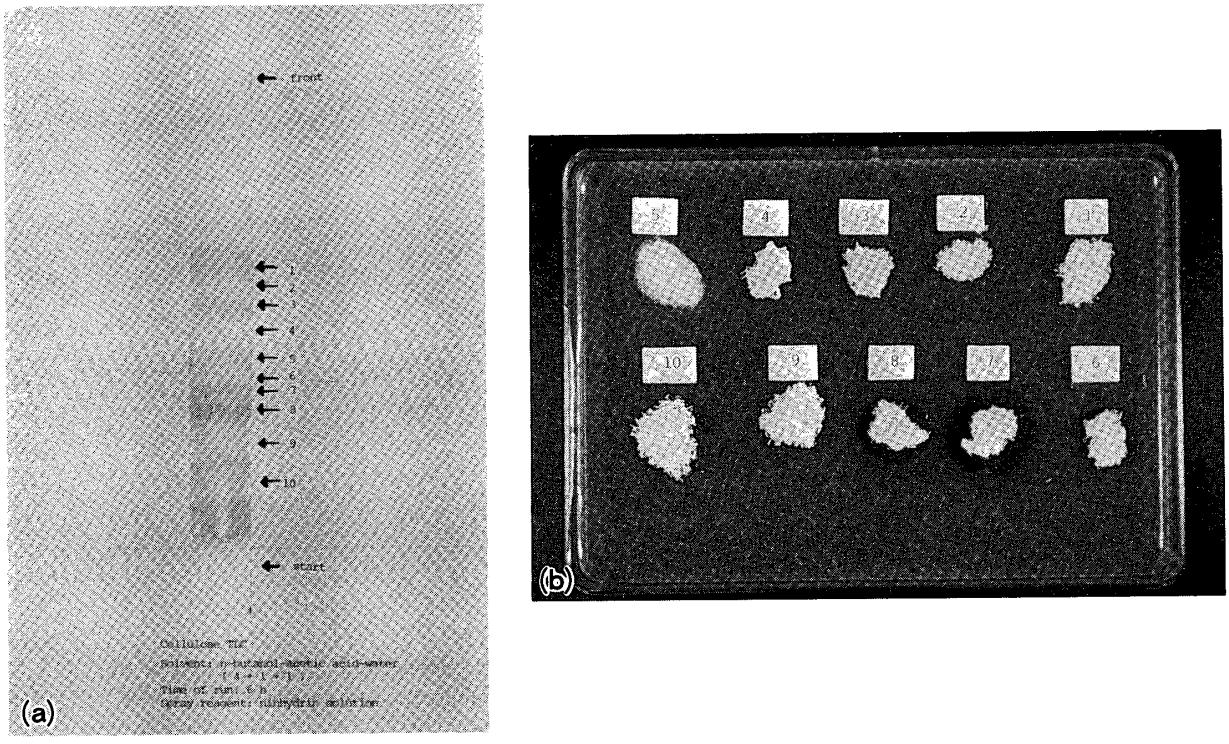

Fig. 9 Cellulose TLC and detection of inhibitory effect of crude ACS. (a) : Distribution of peptide on cellulose-TLC. (b) : Inhibitory effect of various fractions on cellulose-TLC. Procedures were performed as stated in "Materials and methods".

Discussion

There are many identification methods for Candida species[9]; i.e. morphology of colony, chlamydospore formation, glycolysis and acid formation, germtube formation, and serological method. At times, it is difficult to differentiate between *C. albicans* and *C. tropicalis*, because these organisms resemble each other in their characteristics. *C. albicans* differs from *C. tropicalis* in the number of an antigen composition by Candida check[10,11,12], but at times spontaneous agglutination occurs[13]. *C. albicans* forms germtube[14]and chlamydospore, but *C. tropicalis* forms neither. Actually, there are some strains of *C. albicans* in which the characteristics decrease or disappear. *C. tropicalis* produces gas from sucrose and *C. albicans* does not, but there are some strains of *C. tropicalis* which produce gas very weakly. Further, there are some strains of *C. albicans* which weakly produce gas from sucrose. As mentioned above, it is not easy to identify *C. albicans* and *C. tropicalis* readily.

In this study, crude ACS which inhibited the growth of *C. albicans* and *C. stellatoidea*, but did not inhibit the growth of other Candida species was prepared from *B. natto* NA-6. The inhibitory zone was detected in all strains of *C. albicans* by paper disk method, but was not detected in any strains of *C. tropicalis*. There was a clear difference between the two species. Therefore, the paper disk method with crude ACS can be used to readily distinguish between *C. albicans* and *C. tropicalis*. *C. stellatoidea* was also inhibited by crude ACS, but this organism is hardly detected from clinical sources, and few formed chlamydospore and was easily distinguished from *C. albicans* with acid production from sucrose. Kobayashi reported that the character of antifungl substance from *B. subtilis* sb-2w was not identical with Bacillomycin[15], Eumycin[16], Mycosubtilin[17], Subtilin[18], and B-456 substance[19]. These character of crude ACS were identical with the result of her reports[3,4].

On the other hand, *B. natto*[20] is known to closely resemble *B. subtilis* taxonomically[21], Kita et al[22] reported that there are differences between the two organisms in their biotin- or amino acid- requirement. Ushijima and Ozaki[7] also reported that *B. natto* was identified as *B. subtilis* on the basis of Bergey's Manual[21], but these organisms were divided into 2 groups, A and B, with key character of acid production from glycogen, complete hydrolysis of starch and utilization of histidine. In their results, strains isolated from natto belonged to B group, but some strains of *B. natto* belonged to A group and only the strain of A group produced antibacterial substance. In this study, however, all strains of *B. natto* belonged to A group and produced ACS.

Ozawa et al[8] reported that culture filtrate of *B. natto* BN suppressed old stock culture of *C. albicans*, but a fresh isolate from a clinical source did not. In this study, however, both old stock cultures and fresh isolates of *C. albicans* from clinical sources

were inhibited with culture filtrate and crude ACS from *B. natto*. It may be that its difference is due to culture broth where they used Brain Heart infusion, soybean-starch medium was used in this study.

Function of crude ACS against *C. albicans* was mainly bacteriostatic and partially bacteriocidal. This result was also identical with that of Kobayashi, but she did not mention the morphological change of *C. albicans* which was exposed to antifungal substance. In this study, some of *C. albicans* which were exposed to crude ACS swelled at frist, and in a secondary step action were destroyed, but it is too early to speculate on how these two actions are related. This observation was not identical with Mycobacillin[23], which brought about aggregation against the sensitive cells.

Stability of crude ACS was also an important matter for the identification of *C. albicans* at good reproducibility. Crude ACS which was stored at −20℃ and crude ACS solution which was stored at 4°C or −20°C maintained thier original high inhibitory activity for a long time.

In this study, an interesting possibility was seen in the paper disk method with crude ACS which may be useful for the identification of *C. albicans*. Further studies will be required in more detail on the selective inhibitory effect of crude ACS against Candida species.

Conclusion

A crude ACS which was obtained from *B. natto* selectively inhibited the growth of Candida species. By the paper disk method with crude ACS, the inhibitory zone was detected in *C. albicans* and *C. stellatoidea* but was not detected in *C. tropicalis*, *C. pseudotropicalis*, *C. krusei*, *C. parapsilosis* and *C. guillermondii*. MIC of crude ACS against *C. albicans* was 400 μg/ml but *C. tropicalis* was not inhibited at 12,800 μg/ml of crude ACS. The function of crude ACS against *C. albicans* was mainly bacteriostatic and weakly bacteriocidal. These results suggested that the paper disk method with crude ACS was useful method for the identification of *C. albicans*.

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*Bacillus natto*の産生する抗カンジダ物質の阻止作用について

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抄　録

*Bacillus natto*が*Candida albicans*と*C. stellatoidea*に対してはその発育を阻止するが，他の*Candida*種の発育は阻止しない抗真菌物質を菌体外に産生することを確認した。*B. natto*の培養上澄より活性炭の添加と限外ろ過（分子量　10,000）処理により粗抗カンジダ物質（ACS）を得た。粗ACSは供試した*C. albicans*154株の全株の発育を阻止したが，*C. tropicalis*の全株（5株）は発育が阻止されないことをペーパーディスク法により確認した。この方法により，粗ACSはこの2種のカンジダを明確に分別できることが判明した。粗ACSの*C. albicans*に対するMICは400 μg/mlであったが，*C. tropicalis*は12,800 μg/mlでも発育は阻止されなかった。−20℃に保存した粗ACSと4°C又は−20°Cに保存した粗ACS溶液は初期の活性を8週間保持した。粗ACSの*C. albicans*に対する作用は主に静菌的であり，また弱く殺菌的にも作用した。この結果は粗ACSで処理した*C. albicans*の形態変化とも一致した。粗ACS含有サブロー寒天平板上の*C. albicans*の形態は，初めに一部の菌体が膨化し，次いで破壊される像が観察された。また，粗ACS含有液体培地中における*C. albicans*の形態は破壊され遊離した細胞質様物質が菌体周囲に存在し，多くの菌体はグラム陰性に染色された。

以上の成績から，より簡単で迅速かつ正確な*C. albicans*の同定が，このペーパーディスク法と他の同定法とを併用することによって成し得ることが示唆された。

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